BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 39/395, C07K 15/28, C12N 15/13, C12P 21/08 — A1

(11) International Publication Number: WO 94/12215

(43) International Publication Date: 9 June 1994 (09.06.94)

(21) International Application Number: PCT/US93/11612

(22) International Filing Date: 30 November 1993 (30.11.93)

(30) Priority Data:
07/983,946 1 December 1992 (01.12.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/983,946 (CIP)
Filed on 1 December 1992 (01.12.92)

(71) Applicant *(for all designated States except US)*: PROTEIN DESIGN LABS, INC. [US/US]; 2375 Garcia Avenue, Mountain View, CA 94043 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: CO, Man, Sung [GB/US]; 10230 Yoshino Place, Cupertino, CA 95014 (US).

(74) Agents: LIEBESCHUETZ, Joseph et al.; Townsend and Townsend Khourie and Crew, One Market Plaza, 20th floor, Stueart Street Tower, San Francisco, CA 94105 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: HUMANIZED ANTIBODIES REACTIVE WITH L-SELECTIN

(57) Abstract

Humanized immunoglobulins specifically reactive with L-selectin are prepared employing recombinant DNA technology for use in e.g., treatment of inflammatory disorders.

HUMANIZED ANTIBODIES REACTIVE WITH L-SELECTIN

Cross-Reference to Related Inventions

This application is a continuation-in-part of USSN 07/983,946, filed 12/1/92, which is hereby incorporated by reference in its entirety for all purposes.

Field of the Invention

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel biologics and, more particularly, for example, to the production of non-immunogenic (in humans) immunoglobulins specific for the L-selectin protein and their uses *in vitro* and *in vivo*.

Background of the Invention

The ability of cells to adhere to one another plays a critical role in development, normal physiology, and disease processes. This ability is mediated by adhesion molecules, generally glycoproteins, expressed on cell membranes. Often, an adhesion molecule on one cell type will bind to another adhesion molecule expressed on a different cell type, forming a receptor counter-receptor pair. Three very important classes of adhesion molecules are the integrins, selectins, and immunoglobulin (Ig) superfamily members (*see* Springer, *Nature* 346:425 (1990); Osborn, *Cell* 62:3 (1990); Hynes, *Cell* 69:11 (1992), all of which are incorporated herein by reference in their entirety for all purposes). These molecules are especially vital to the interaction of leukocytes and platelets with themselves and with the extracellular matrix and vascular endothelium.

Integrins are heterodimeric transmembrane glycoproteins consisting of an α chain (120-180 kD) and a β chain (90-110 kD), generally having short cytoplasmic domains. The α subunits all share sequence homology and motifs with each other, as do the β subunits. The three

receptors as well. In particular, E-selectin binds the carbohydrate group sialyl Lewis x (sLex) (Lowe et al., *Cell* 63:475 (1990)), which is incorporated herein by reference in its entirety for all purposes), and while this carbohydrate is prominently presented on L-selectin (Picker et al., *Cell* 66:921 (1991)), it may occur on other proteins as well. E-selectin is expressed especially in cutaneous sites of inflammation and also serves as an adhesion molecule for skin-homing T cells that may contribute to the inflammation (Picker et al., *Nature* 349:796 (1991), which is incorporated herein by reference in its entirety for all purposes).

In various assays, antibodies to CD11a, CD11b, CD18, L-selectin and E-selectin all block binding of neutrophils to activated endothelial cells to a lessor or greater degree, but the most complete inhibition is generally achieved by the combination of an antibody to CD18 and an antibody to L- or E-selectin (*see, e.g.*, Luscinskas, *J. Immunol.* 142:2257 (1989)), which is incorporated herein by reference in its entirety for all purposes). A recent but now widely accepted model accounts for these facts with a three step process of adhesion (Butcher, *Cell* 67:1033 (1991), which is incorporated herein by reference in its entirety for all purposes). In the first step, neutrophils reversibly bind to inflamed vascular endothelium via the selectins, which bind well under conditions of flow, causing the neutrophils literally to roll along the vascular wall. The neutrophils are then activated by a variety of stimulants surrounding or released by the endothelium, including IL-8, PAF and C5a. The activated neutrophils shed L-selectin and up-regulate Mac-1. In the final step, binding of Mac-1 to ICAM-1 and perhaps other counter-receptors on the endothelial cells allows stable adhesion and extravasation through the endothelium.

In principle, antibodies or other antagonists of the integrin and selectin adhesion molecules could abort this process, by preventing neutrophils from binding to endothelium and from extravasating into tissues. Hence such antibodies could be used to treat a great many different

partially or completely block the binding of human lymphocytes to peripheral lymph node high endothelial venules, and the binding of human neutrophils to stimulated human umbilical vein endothelial cells (Kishimoto et al., *Blood* 78:805 (1991), which is incorporated herein by reference in its entirety for all purposes). The capacity of these antibodies to block binding of neutrophils to endothelial cells indicates that the antigen to which they bind, L-selectin, may be an appropriate target for potential therapeutic agents.

Unfortunately, the use of non-human monoclonal antibodies such as mouse DREG-200 have certain drawbacks in human treatment, particularly in repeated therapeutic regimens as explained below. Mouse monoclonal antibodies, for example, have a relatively short circulating half-life, and lack other important immunoglobulin functional characteristics when used in humans.

Perhaps more importantly, non-human monoclonal antibodies contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that, after injection of a foreign antibody, the immune response elicited by a patient against an antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, as increasing numbers of different mouse or other antigenic (to humans) monoclonal antibodies can be expected to be developed to treat various diseases, after the first or several treatments with any different non-human antibodies, subsequent treatments even for unrelated therapies can be ineffective or even dangerous in themselves, because of cross-reactivity. While the production of so-called "chimeric antibodies" (*e.g.*, mouse variable regions joined to human constant regions) has proven somewhat successful, a significant immunogenicity problem remains.

To attempt to overcome immunogenicity problems several examples of humanized antibodies have been produced. The transition from a murine to a humanized antibody involves

produce humanized immunoglobulins capable of binding to the L-selectin at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to L-selectin.

The immunoglobulins, including binding fragments and other derivatives thereof, of the present invention may be produced readily by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a first sequence coding for humanized immunoglobulin framework regions and a second sequence set coding for the desired immunoglobulin complementarity determining regions can be produced synthetically or by combining appropriate cDNA and genomic DNA segments.

The humanized immunoglobulins may be utilized alone in substantially pure form, or together with a chemotherapeutic agent such as a non-steroidal anti-inflammatory drug (e.g., aspirin), a corticosteroid, or an immunosuppressant. All of these compounds will be particularly useful in treating inflammatory disorders. The humanized immunoglobulins or their complexes can be prepared in a pharmaceutically acceptable dosage form, which will vary depending on the mode of administration.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Sequences of the CDNA and translated amino acid sequences of the light chain (A) and heavy chain (B) variable regions of the mouse DREG-200 antibody. The mature heavy chain begins with amino acid 20 E, and the mature light chain begins with amino acid 21 D, preceded by the respective signal sequences.

Figure 2. Amino acid sequences of the mature light chain (A) and heavy chain (B) variable regions of the mouse DREG-200 antibody (upper lines) and humanized DREG-200 antibody (lower lines). The three CDRs in each chain are underlined. Residues in the framework that have been

preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

Amino acids from the variable regions of the mature heavy and light chains of immunoglobulins are designated Hx and Lx respectively, where x is a number designating the position of an amino acids according to the scheme of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991). Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. Kabat's scheme is extendible to other antibodies not included in his compendium by aligning the antibody in question with one of the consensus sequences in Kabat. The use of the Kabat numbering system readily identifies amino acids at equivalent positions in different antibodies. For example, an amino acid at the L50 position of a human antibody occupies the equivalent position to an amino acid position L50 of a mouse antibody.

From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in

large quantities, find use, for example, in the treatment of inflammatory disorders in human patients by a variety of techniques.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The NH_2-terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The COOH part of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (*See, generally, Fundamental Immunology*, Paul, W., Ed., Chapter 7, pp. 131-166, Raven Press, N.Y. (1984), which is incorporated herein by reference in its entirety for all purposes.)

The variable regions of each light/heavy chain pair form the antibody binding site. The chains all exhibit the same general structure of relatively conserved framework regions joined by three hypervariable regions, also called Complementarity Determining Regions or CDRs (*see* "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services (1987); and Chothia and Lesk, *J. Mol. Biol.*, 196:901-917 (1987), which are incorporated herein by reference in their entirety for all purposes). The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The

constant region present is substantially identical to a human immunoglobulin constant region, *i.e.*, at least about 85-90%, preferably at least 95% identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. For example, a humanized immunoglobulin would not encompass a chimeric mouse variable region/human constant region antibody.

Humanized antibodies have at least three potential advantages over mouse, and in some cases chimeric antibodies, for use in human therapy:

1) because the effector portion is human, it may interact better with the other parts of the human immune system (*e.g.*, destroy the target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC)).
2) The human immune system should not recognize the framework or C region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.
3) Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal antibodies (Shaw, D. et al., *J. Immunol.* 138:4534-4538 (1987)). Injected humanized antibodies will presumably have a half-life more like that of naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDRs from an immunoglobulin capable of binding to a desired epitope of L-selectin, such as monoclonal antibodies mouse DREG-200, mouse DREG-55 or mouse DREG-56 (Kishimoto et

Publication No. 0239400 and Riechmann, L. et al., *Nature* 332:323-327 (1988), both of which are incorporated herein by reference in their entirety for all purposes).

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (*see* Kabat, *supra*, and WP87/02671). The CDRs for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to L-selectin and produced in any convenient mammalian source, including, mice, rats, rabbits, or other vertebrate capable of producing antibodies by well known methods. Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection (*Catalogue of Cell Lines and Hybridomas*, Fifth edition (1985) Rockville, MD, which is incorporated herein by reference in its entirety for all purposes). In preferred embodiments, the CDRs have sequences corresponding to the CDR sequences of mouse DREG-200, mouse DREG-55, or mouse DREG-56, respectively, and may include degenerate nucleotide sequences encoding the corresponding CDR amino acid sequence(s) of mouse DREG-200, mouse DREG-55, or mouse DREG-56.

In addition to the humanized immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. Other human antibodies than the Eu antibody discussed in Example 2 can be used as a source of framework sequence. These framework sequences should exhibit a high degree of sequence identity with the mouse DREG-200 variable framework domains from which the CDRs were derived. The heavy and light chain variable framework regions can be derived from the same or different human antibody sequences. Indeed, the heavy and light chain framework regions can each be derived from more than one human antibody. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be

antibody eliciting a HAMA response in humans. Amino acids are selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is by modelling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids.

When an amino acid differs between a mouse DREG-200 variable framework region and an equivalent human variable framework region, the human framework amino acid should usually be substituted by the equivalent mouse amino acid if it is reasonably expected that the amino acid:

(1) noncovalently contacts antigen directly, or

(2) is adjacent to a CDR region or otherwise interacts with a CDR region (*e.g.*, is within about 4-6 Å of a CDR region).

Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position (*e.g.*, amino acid H113 of human Eu antibody). These amino acids can be substituted with amino acids from the equivalent position of more typical human immunoglobulins. Alternatively, amino acids from equivalent positions in the mouse DREG-200 can be introduced into the human framework regions when such amino acids are typical of human immunoglobulin at the equivalent positions.

In general, substitution of all or most of the amino acids fulfilling the above criteria is desirable. Occasionally, however, there is some ambiguity about whether a particular amino acid meets the above criteria, and alternative variant immunoglobulins are produced, one of which has that particular substitution, the other of which does not. The humanized antibodies of the present invention will usually contain a substitution with a mouse light chain framework residue with a corresponding mouse DREG-200 residue in at least 1, 2, 3, 4 and more usually 5, of the following positions: L87, L54, L66, L76 and L93. The humanized antibodies also usually contain a substitution with a mouse heavy chain framework residue in at least 1, 3, 5, 7, 9, 10,

accomplished by a variety of well-known techniques, such as site-directed mutagenesis (*see* Gillman & Smith, *Gene* 8:81-97 (1979) and Roberts et al., *Nature* 328:731-734 (1987), both of which are incorporated herein by reference in their entirety for all purposes).

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (*e.g.*, binding activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in the vectors pVk and pVg1-dhfr using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce $(Fab')_2$ fragments. Single chain antibodies may be produced by joining VL and VH with a DNA linker (*see* Huston et al., *supra*, and Bird et al., *supra*). As one example, Fv or Fab fragments may be produced in *E. coli* according to the methods of Buchner and Rudolph, *Bio/Technology* 9:157-162 (1991) and Skerra et al., *Bio/Technology* 9:273-277 (1991), incorporated herein by reference in their entirety for all purposes. Fv and Fab may also be produced by expression of encoding polynucleotides in eukaryotic, preferably mammalian, cells. Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes (*e.g.*, enzymes, *see* commonly assigned U.S.S.N. 132,387, filed Dec. 15, 1987, which is incorporated herein by reference in its entirety for all purposes) to produce fusion proteins (*e.g.*, immunotoxins) having novel properties.

Expression of the humanized immunoglobulin sequences in bacterial hosts may be used to advantage to select higher affinity humanized immunoglobulin sequences by mutagenizing the CDR regions and producing bacteriophage display libraries which may be screened for humanized immunoglobulin CDR variants which possess high affinity and/or high specificity binding to L-selectin. One potential

system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. *Saccharomyces* is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

Plants and plant cell cultures may be used for expression of the humanized immunoglobulins of the invention. (Larrick & Fry, *Hum. Antibodies Hybridomas* 2(4):172-89 (1991); Benvenuto et al., *Plant Mol. Biol.* 17(4):865-74 (1991); Durin et al., *Plant Mol. Biol.* 15(2):281-93 (1990); Hiatt et al., *Nature* 342:76-8 (1989), incorporated herein by reference in their entirety for all purposes). Preferable plant hosts include, for example: *Arabidopsis*, *Nicotiana tabacum*, *Nicotiana rustica*, and *Solanum tuberosum*. A preferred expression cassette for expressing polynucleotide sequences encoding the humanized anti-L-selectin antibodies of the invention is the plasmid pMOG18 in which the ins rted polynucleotide sequence encoding the humanized immunoglobulin chain is operably linked to a CaMV 35S promoter with a duplicated enhancer; pMOG18 is used according to the method of Sijmons et al., *Bio/Technology* 8:217-221 (1990), incorporated herein by reference in its entirety for all purposes. Alternatively, a preferred embodiment for the expression of humanized immunoglobulins in plants follows the methods of Hiatt et al., *supra*, with the substitution of polynucleotide sequences encoding the humanized anti-L-selectin antibodies of the invention for the immunoglobulin sequences used by Hiatt et al., *supra*. *Agrobacterium tumifaciens* T-DNA-based vectors may also be used for expressing humanized immunoglobulin sequences, preferably

animals which express the desired humanized immunoglobulin, typically in a recoverable body fluid such as milk or serum. Such transgenes comprise a polynucleotide sequence encoding the humanized immunoglobulin(s) operably linked to a promoter, usually with a linked enhancer, such as a rodent immunoglobulin enhancer or a casein gene promoter/enhancer (Buhler et al., *Bio/Technology* 8:140-143 (1990); Meade et al., *Bio/Technology* 8:443-446 (1990), incorporated herein by reference in its entirety for all purposes). Transgenes may be transferred into cells and embryos according to the methods described in the art and, *infra*, for homologous recombination constructs. Preferred nonhuman animals include: mice, rats, sheep, cows, and goats; with expression in bovine milk being particularly preferred. *See* WO91/08216 (1991) (which is incorporated in its entirety for all purposes). Purification of the humanized antibodies is accomplished by art-known purification methods for immunoglobulin purification.

The vectors containing the DNA segments of interest (*e.g.*, the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, lipofection, biolistics, viral-based transduction, or electroporation may be used for other cellular hosts. Tungsten particle ballistic transgenesis is preferred for plant cells and tissues. (*See, generally*, Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, 1982), which is incorporated herein by reference in its entirety for all purposes.)

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see, generally*, Scopes, R.,

Corp. USA) is suitable. Computers are useful for generating variants of humanized antibodies. In general, the antibodies of the invention already provide satisfactory binding affinity. However, it is likely that antibodies with even stronger binding affinity could be identified by further variation of certain amino acid residues. The three dimensional image will also identify many noncritical amino acids, which could be the subject of conservative substitutions without appreciable affecting the binding affinity of the antibody. Collectively even conservative substitutions can have a significant effect on the properties of an immunoglobulin. However, it is likely many individual conservative substitutions will not significantly impair the properties of the immunoglobulins.

Human Antibodies Against L-Selectin

In another aspect of the invention, human antibodies against L-selectin are provided. These antibodies are produced by a variety of techniques described below. Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody, such as mouse DREG-200 or a humanized version thereof. Such antibodies are particularly likely to share the useful therapeutic properties demonstrated for humanized DREG-200.

Antibodies having the required epitope specificity can also be identified by screening for the capacity to block neutrophil-endothelial cell interaction. A simple visual assay for detecting such interaction has been described by Kishimoto et al. (1991), *supra*. Briefly, monolayers of human umbilical vein cells are stimulated with IL-1. Neutrophils, with or without pretreatment with the antibody under test, are added to the monolayer under defined conditions, and the number of adhering neutrophils is determined microscopically. In one method, the neutrophils are obtained from human leukocyte adhesion deficient patients. *See* Anderson et al., *Ann. Rev. Med.* 38:175 (1987). The neutrophils from such patients lack integrin receptors, whose binding to

HAT or AH). Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to L-selectin or a fragment thereof. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown *in vitro* in culture medium. The trioma cell lines obtained are then tested for the ability to bind L-selectin or a fragment thereof.

Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into a cell line such as the cell lines discussed *supra* for expression of recombinant or humanized immunoglobulins.

b. Transgenic Non-Human Mammals

Human antibodies against L-selectin can also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus. Usually, the endogenous immunoglobulin locus of such transgenic mammals is functionally inactivated. Preferably, the segment of the human immunoglobulin locus includes unrearranged sequences of heavy and light chain components. Both inactivation of endogenous immunoglobulin genes and introduction of exogenous immunoglobulin genes can be achieved by targeted homologous recombination, or by introduction of YAC chromosomes. The transgenic mammals resulting from this process are capable of functionally rearranging the immunoglobulin component sequences, and expressing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes, without expressing endogenous immunoglobulin genes. The production and properties of mammals having these properties are described in detail by, e.g., Lonberg et al., WO93/12227 (1993); Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in its entirety for all purposes). Transgenic mice are particularly suitable. Anti-L-selectin antibodies are obtained by immunizing a transgenic nonhuman

as a starting material for constructing a further phage library. In this library, each phage displays the same heavy chain variable region (*i.e.*, the region identified from the first display library) and a different light chain variable region. The light chain variable regions are obtained from a library of rearranged human variable light chain regions. Again, phage showing strong specific binding for L-selectin are selected. These phage display the variable regions of completely human anti-L-selectin antibodies. These antibodies usually have the same or similar epitope specificity as the murine starting material (*e.g.*, mouse DREG-200).

Methods of Use

The antibodies of the present invention will typically find use in the treatment of disease conditions with an inflammatory component, especially those which are mediated by neutrophils or T cells. A preferred application is the therapeutic and prophylactic treatment of ischemia-reperfusion injury caused by myocardial infarction, cerebral ischemic event (*e.g.*, stroke), renal, hepatic or splenal infarction, brain surgery, shock, cardiac surgery (*e.g.*, coronary artery bypass), elective angioplasty, and the like. Other preferred applications are the treatment of sepsis, adult respiratory distress syndrome, and multiple organ failure. The antibodies will find use in treating injury due to trauma, burns, frostbite or damage to the spinal cord. They will also find use in treating autoimmune diseases including by way of example and not limitation, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, type I diabetes and uveitis, in treating inflammatory diseases of the skin such as psoriasis, and in treating meningitis and encephalitis. Other typical applications are the prevention and treatment of organ transplant rejection and graft-versus-host disease.

Any immunoglobulin of the present invention may also be used in combination with other antibodies, particularly humanized or human antibodies reactive with

treatment is usually 5 - 30 mins, preferably 5 - 20 min, and most preferably 5 - 10 min. The antibodies are administered parentally, preferably by intravenous injection, in doses of 0.01 - 10 mg/kg body weight, preferably of 0.14 - 5 mg/kg and most preferably of 0.3 - 3 mg/kg. The antibodies can be given as an intravenous bolus injection, *e.g.*, over 1 - 5 min., as repeated injections of smaller doses, or as an intravenous infusion. The bolus injection is especially useful for the prophylactic dose or in an emergency. Further doses of antibodies can be repeated (*e.g.*, every 4 - 6 h) during and after thrombolytic or angioplastic treatment of acute myocardial infarction at the same proportions as described above to achieve optimal plasma levels of the antibody.

Thrombolytic agents are drugs having the capacity, directly or indirectly, to stimulate dissolution of thrombi *in vivo*. Thrombolytic agents include tissue plasminogen activator (*see* EP-B 0 093 619), activase, alteplase, duteplase, silteplase, streptokinase, anistreplase, urokinase, heparin, warfarin and coumarin. Additional thrombolytic agents include saruplase and vampire bat plasminogen activator. *See* Harris, *Protein Engineering* *6*:449-458 (1987); PCT/EP 90/00194; US Patent 4,970,159). Thrombolytic agents are administered to a patient in an amount sufficient to partially disperse, or prevent the formation of, thrombi and their complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "efficacious dose." Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, the route of administration and combination with other drugs. Often, therapeutically effective doses of thrombolytic agents and administration regimens for such agents are those approved by the FDA, for independent uses of thrombolytic agents, *e.g.*, 100 mg of alteplase or 1.5 million IU of streptokinase.

A preferred pharmaceutical composition of the present invention comprises the use of the subject immunoglobulins in immunotoxins to kill L-selectin expressing

incorporated herein by reference in their entirety for all purposes.)

The delivery component of the immunotoxin will include the immunoglobulins of the present invention. Intact immunoglobulins or their binding fragments, such as Fab or Fv, are preferably used. Typically, the antibodies in the immunotoxins will be of the human IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The antibodies and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration, *i.e.*, subcutaneously, intramuscularly or intravenously. The antibodies of the invention may also be administered, typically for local application, by gavage or lavage, intraperitoneal injection, ophthalmic ointment, topical ointment, intracranial injection (typically into a brain ventricle), intrapericardiac injection, or intrabursal injection. The compositions for parenteral administration will commonly comprise a solution of the immunoglobulin or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, water, buffered water, phosphate buffered saline (PBS), 0.4% saline, 0.3% glycine, human albumin solution and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride and sodium lactate. The concentration of antibody in these formulations can vary widely, *i.e.*, from less than about 0.005%, usually at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

commonly used. Dosing schedules will vary with the disease state and status of the patient, and will typically range from a single bolus dosage or continuous infusion to multiple administrations per day (*e.g.*, every 4-6 hours), or as indicated by the treating physician and the patient's condition. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases, in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the immunoglobulins of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already suffering from a particular disease to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 1 to 70 mg per dose. Preferred prophylactic uses are for the prevention of adult respiratory distress syndrome in patients already suffering from sepsis or trauma; prevention of organ transplant rejection; and prevention of reperfusion injury in patients suffering from ischemia. In seriously ill patients, dosages of about 50 to 150 mg of humanized or human immunoglobulin per administration are frequently used, and larger dosages may be indicated.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

Antibodies of the present invention can further find a wide variety of utilities *in vitro*. By way of

hybridized to the dG tails and contained EcoRI sites. The PCR amplified fragments were digested with EcoRI and HindIII and cloned into the pUC18 vector for sequencing. For mouse DREG-200, at least two gamma-1 specific and two kappa specific clones were sequenced. The gamma-1 clones and the kappa clones are respectively identical in sequence. The cDNA variable domain sequences and the deduced amino acid sequences are shown in Fig. 1.

Example 2: Computer Modeling of Humanized Antibodies.

In order to retain high binding affinity in the humanized antibodies, the general procedures of Queen et al. were followed (*see* Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029 (1989) and WO 90/07861, which are incorporated herein by reference in their entirety for all purposes). The more homologous an acceptor human antibody is to the original murine donor antibody, the less likely will combining the murine CDRs with the human framework be to introduce distortions into the CDRs that could reduce affinity. Homology (that is, percent sequence identity) of at least 65% between the humanized immunoglobulin heavy chain variable region framework and the donor immunoglobulin heavy chain variable region framework is preferred. Normally the heavy chain and light chain from the same human antibody are chosen to provide the framework sequences, so as to reduce the possibility of incompatibility in the assembling of the two chains. Based on sequence homology search against the NBRF protein sequence database (performed with the MicroGeni Sequence Analysis Software (Beckman)), the antibody Eu was chosen to provide the framework sequences for humanization of mouse DREG-200.

The computer program ENCAD (Levitt, *J. Mol. Biol.* 168:595 (1983), which is incorporated herein by reference in its entirety for all purposes) was used to construct a model of the mouse DREG-200 variable region. The model was used to determine the amino acids in the mouse DREG-200 framework that were close enough to the CDRs to potentially interact with them (category 4 below). To design the humanized light

TABLE 1

Category	Light Chain	Heavy Chain
1	24-40, 56-62, 95-103	31-35, 50-66, 99-110
2	87	93, 95, 98, 111, 112, 115
3	--	30, 98, 111
4	54, 66, 76, 93	27, 30, 48, 72
5	--	113

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, including signal peptides, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences of the genes also included splice donor signals and an XbaI site at each end. The nucleotide sequences and encoded humanized light and heavy chain variable domains are shown in Fig. 3. Each gene was constructed from four overlapping synthetic oligonucleotides, as described (*see* Co et al., *J. Immunol.* 148:1149 (1992), and commonly assigned U.S.S.N. 07/634,278, which are incorporated herein by reference in their entirety for all purposes.) The heavy and light chain variable region genes were then respectively ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors (*see* commonly assigned U.S.S.N 07/634,278) in the appropriate orientations to produce the complete heavy and light chain genes. Reactions were carried out under conditions well-known in the art (Maniatis et al., *supra*)

The heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells were selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ELISA, and antibody was purified from the best-producing clones. Humanized DREG-200 IgG1 antibody was then purified by passing tissue culture supernatant over a column

assay method of Hallmann et al., *Biochem. Biophys. Res. Comm.* 174:236 (1991). Specifically, human umbilical cord endothelial cells (HUVEC; from Clonetics, San Diego) were grown to confluence in EGM medium (Clonetics) in Lab-Tek 8-chamber slides (Nunc, Naperville, IL). The HUVEC cells were stimulated with 20 ng/ml IL-1β (R&D Systems, Minneapolis, MN) for 4 hr before use. Neutrophils were isolated by density gradient centrifugation from buffy coats that had been cleared of erythrocytes by dextran sedimentation, and then adjusted to 10^7 per ml. The neutrophils (100 μl) were pre-incubated for 20 minutes on ice with varying concentrations of antibody (in 100 μl RPMI). The HUVEC slides were washed free of IL-1β and placed on a rotary shaker (100 rpm) at 4°C. The untreated or antibody treated neutrophils were added to the chambers, and the slide was incubated at 4°C on the shaker for 30 min. The slides were then washed by dipping ten times into a beaker of RPMI, fixed in 1% glutaraldehyde in RPMI, and allowed to air dry. Neutrophil adherence was quantified by counting the neutrophils attached to a defined area of the endothelial cell monolayer with a microscope. As shown in Fig. 5, the humanized IgG1 and mouse DREG-200 antibodies both effectively blocked the binding of neutrophils to the HUVEC, while an irrelevant control antibody did not. The humanized DREG-200 IgG4 antibody will similarly block binding of neutrophils to endothelial cells.

Example 4: Effect of hu DREG-200 on Myocardial Injury Following Reperfusion.

The effect of humanized DREG-200 of the IgG4 isotype (hu DREG-200) on the degree of actual salvage of myocardial ischemic tissue following reperfusion was investigated. Adult male cats (2.8-4.2 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). An intratracheal cannula was inserted through a midline incision, and the cats were placed on intermittent positive-pressure ventilation (Harvard small animal respirator, Dover, MA). A polyethylene catheter was inserted into the right external jugular vein for additional pentobarbital infusion in order to maintain a surgical plane

The cats were randomly divided into three major groups. Six sham MI + R cats received hu DREG-200 (2 mg/kg), six MI + R cats received the control MAb hu ABL-364 (2 mg/kg), and six MI + R cats received hu DREG-200 (2 mg/kg). Sham MI + R cats were subjected to the same surgical procedures as MI + R cats except that the LAD coronary artery was not occluded.

At the end of the 4.5 h reperfusion period, the ligature around the LAD was again tightened. 20 ml of 0.5% Evans blue was rapidly injected into the left ventricle to stain the area of myocardium which was perfused by the patent coronary arteries. The area-at-risk was determined by negative staining. Immediately following this injection, the heart was rapidly excised and placed in warmed, oxygenated K-H solution. The left circumflex (LCX) and the LAD coronary arteries were isolated and removed for subsequent study of coronary ring vasoactivity and PMN adherence. The right ventricle, great vessels, and fat tissue were carefully removed, and the left ventricle was sliced parallel to the atrioventricular groove in 3 mm thick sections. The unstained portion of the myocardium (*i.e.*, the total area-at-risk or ischemic area) was separated from the Evans blue stained portion of the myocardium (*i.e.*, the area-not-at-risk or nonischemic area). The area-at-risk was sectioned into small cubes and incubated in 0.1% nitroblue tetrazolium in phosphate solution at pH 7.4 and 37°C for 15 min. The tetrazolium dye forms a blue formazan complex in the presence of myocardial cells containing active dehydrogenases and their cofactors. The irreversibly injured or necrotic portion of the myocardium-at-risk, which did not stain, was separated from the stained portion of the myocardium (*i.e.*, the ischemic but non-necrotic area). The three portions of the myocardium (*i.e.*, non-ischemic, ischemic non-necrotic, and ischemic necrotic tissue) were subsequently weighed. Results were expressed as necrotic cardiac tissue area as a percentage of either the area-at-risk or of total left ventricular mass.

According to both of these criteria, cardiac tissue damage was significantly attenuated ($p<0.001$) in cats treated with hu DREG-200. Whereas about 30% of the

Example 5: Effect of hu DREG-200 on Cardiac Function.

The effect of hu DREG-200 (IgG4 isotype) on cardiac function was determined by measurement of left ventricular pressure (LVP), and the first derivative of LVP, dP/dt max, an index of myocardial contractility. Data were obtained from a catheter tip manometer inserted in the left ventricular cavity. The three groups of cats discussed in the previous example all showed comparable initial values for these cardiac variables. In the sham MI group there were no significant changes in dP/dt max over the entire six hour experimental period. However, in both MI/R groups, dP/dt max decreased upon occlusion of the LAD to about 65%. In cats given hu ABL-364, contractility did not significantly recover. However, in hu DREG-200 treated MI-R cats, dP/dt max recovered to control values three hours following reperfusion. Hence, after 4.5 hours of reperfusion, dP/dt max was significantly lower in hu ABL-364 treated cats than in hu DREG-200 treated cats ($p<0.01$). These results indicate that hu DREG-200 not only reduced myocardial necrosis following reperfusion of the ischemic myocardium, but this myocardial salvage was also translated into an improvement in mechanical performance of the heart.

From the foregoing, it will be appreciated that the immunoglobulins of the present invention offer numerous advantages over other L-selectin specific antibodies. In comparison to mouse monoclonal antibodies, the present immunoglobulins can be more economically produced and contain substantially less foreign amino acid sequences. This reduced likelihood of antigenicity after injection into a human patient represents a significant therapeutic improvement.

All publications and patent applications cited above are herein incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent application were specifically and individually indicated to be so incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purpos s of clarity and

WE CLAIM:

1. A humanized immunoglobulin having complementarity determining regions (CDRs) corresponding to CDRs from a donor immunoglobulin and heavy and light chain variable region frameworks corresponding to human acceptor immunoglobulin heavy and light chain frameworks, which humanized immunoglobulin specifically binds to human L-selectin with an affinity constant of at least 10^7 M^{-1}, wherein the sequence of the humanized immunoglobulin heavy chain variable region framework is 65% or more identical to the sequence of the donor immunoglobulin heavy chain variable region framework.

2. A humanized immunoglobulin according to claim 1 which is an antibody comprising two light chain/heavy chain dimers.

3. A humanized immunoglobulin of claim 2, wherein said antibody is of the IgG1 or IgG4 isotype.

4. A humanized immunoglobulin according to claim 1, which specifically binds to human L-selectin with an affinity of at least 10^8 M^{-1}.

5. A humanized immunoglobulin according to claim 1, wherein said donor immunoglobulin is the mouse DREG-200 antibody.

6. A humanized immunoglobulin according to claim 1, wherein said acceptor immunoglobulin heavy and light chain frameworks are from the same human antibody.

7. A humanized immunoglobulin according to claim 6, wherein said human antibody is the Eu human antibody.

donor immunoglobulin framework replacing the corresponding amino acids in the acceptor immunoglobulin heavy or light chain frameworks, said amino acids not in positions 26-30 of the heavy chain, and each of said amino acids:

(i) is adjacent to a CDR in the donor immunoglobulin sequence, or

(ii) contains an atom within a distance of 4 angstroms of a CDR in said humanized immunoglobulin.

11. A humanized immunoglobulin according to claims 9 or 10 wherein the distance from said atom to said CDR is determined from a computer-generated model of an immunoglobulin.

12. A humanized immunoglobulin according to claims 9 or 10, wherein said donor immunoglobulin is the mouse DREG-200 antibody.

13. A humanized immunoglobulin according to claim 9 which is an antibody comprising two light chain/heavy chain dimers.

14. A humanized immunoglobulin of claim 13, wherein said antibody is of the IgG1 or IgG4 isotype.

15. A humanized immunoglobulin according to claim 9, wherein said acceptor immunoglobulin heavy and light chain frameworks are both from the Eu human antibody.

16. A humanized immunoglobulin according to claims 1 or 9 which is substantially pure.

17. A humanized immunoglobulin according to claims 1 or 9 that inhibits the binding of human neutrophils to human endothelial cells.

18. A composition comprising a humanized immunoglobulin according to claims 1 or 9.

27. A method according to claim 26, further comprising the step of administering a therapeutically effective dose of a thrombolytic agent.

28. A method according to claim 27, wherein the ischemia-reperfusion injury is due to myocardial infarction or balloon angioplasty.

29. A humanized immunoglobulin of claim 1 comprising a humanized heavy chain and a humanized light chain:

(1) the humanized light chain comprising three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse DREG-200 immunoglobulin light chain, and having a variable region framework from a human light chain variable region framework sequence except in at least one position selected from a first group consisting of L87, L54, L66, L76 and L93, wherein said amino acid position is occupied by the same amino acid present in the equivalent position of the mouse DREG-200 immunoglobulin light chain variable region framework; and

(2) the humanized heavy chain comprising three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse DREG-200 immunoglobulin heavy chain, and having a variable region framework from a human heavy chain variable region framework sequence except in at least one position selected from a group consisting of H93, H95, H98, H111, H112, H115, H30, H98, H111, H27, H30, H48 and H72, wherein said amino acid position is occupied by the same amino acid present in the equivalent position of the mouse DREG-200 immunoglobulin heavy chain variable region framework;

wherein the immunoglobulin binds to a L-selectin ligand with a binding affinity that is within three-fold of the binding affinity of the mouse DREG-200 immunoglobulin.

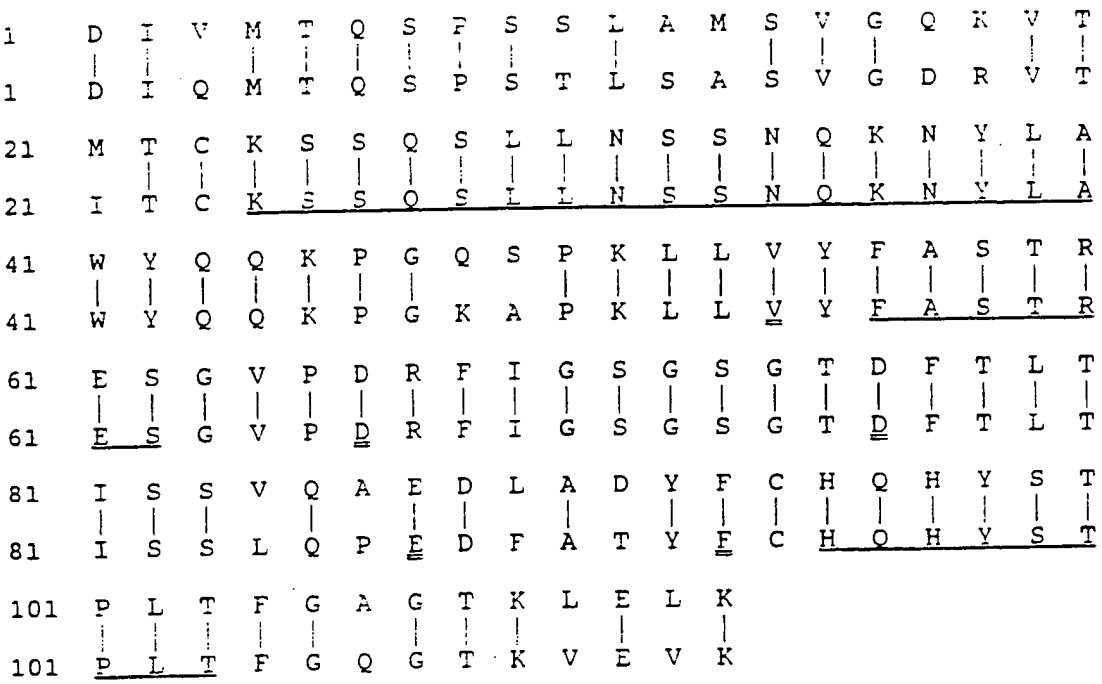
1 D I V M T Q S P S S L A M S V G Q K V T
1 D I Q M T Q S P S T L S A S V G D R V T
21 M T C K S S Q S L L N S S N Q K N Y L A
21 I T C K S S Q S L L N S S N Q K N Y L A
41 W Y Q Q K P G Q S P K L L V Y F A S T R
41 W Y Q Q K P G K A P K L L V Y F A S T R
61 E S G V P D R F I G S G S G T D F T L T
61 E S G V P D R F I G S G S G T D F T L T
81 I S S V Q A E D L A D Y F C H Q H Y S T
81 I S S L Q P E D F A T Y F C H Q H Y S T
101 P L T F G A G T K L E L K
101 P L T F G Q G T K V E V K

FIG. 2A

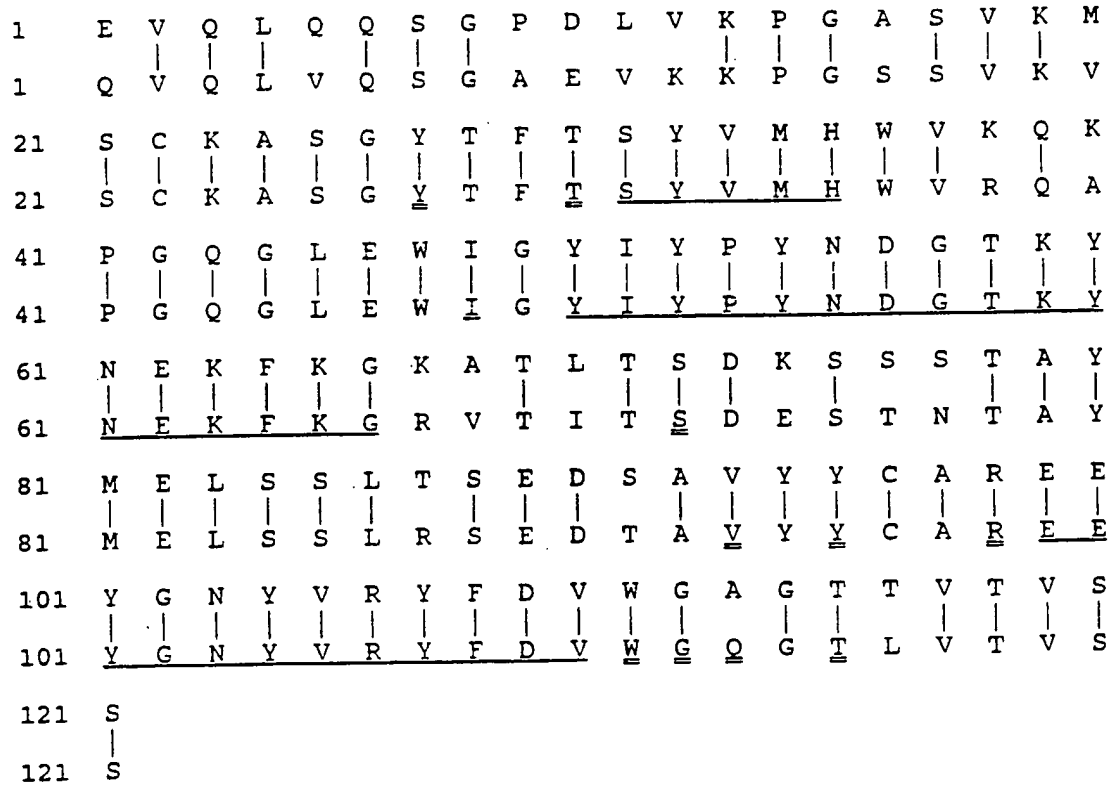
1 E V Q L Q Q S G P D L V K P G A S V K M
1 Q V Q L V Q S G A E V K K P G S S V K V
21 S C K A S G Y T F T S Y V M H W V K Q K
21 S C K A S G Y T F T S Y V M H W V R Q A
41 P G Q G L E W I G Y I Y P Y N D G T K Y
41 P G Q G L E W I G Y I Y P Y N D G T K Y
61 N E K F K G K A T L T S D K S S S T A Y
61 N E K F K G R V T I T S D E S T N T A Y
81 M E L S S L T S E D S A V Y Y C A R E E
81 M E L S S L R S E D T A V Y Y C A R E E
101 Y G N Y V R Y F D V W G A G T T V T V S
101 Y G N Y V R Y F D V W G Q G T L V T V S
121 S
121 S

FIG. 2B

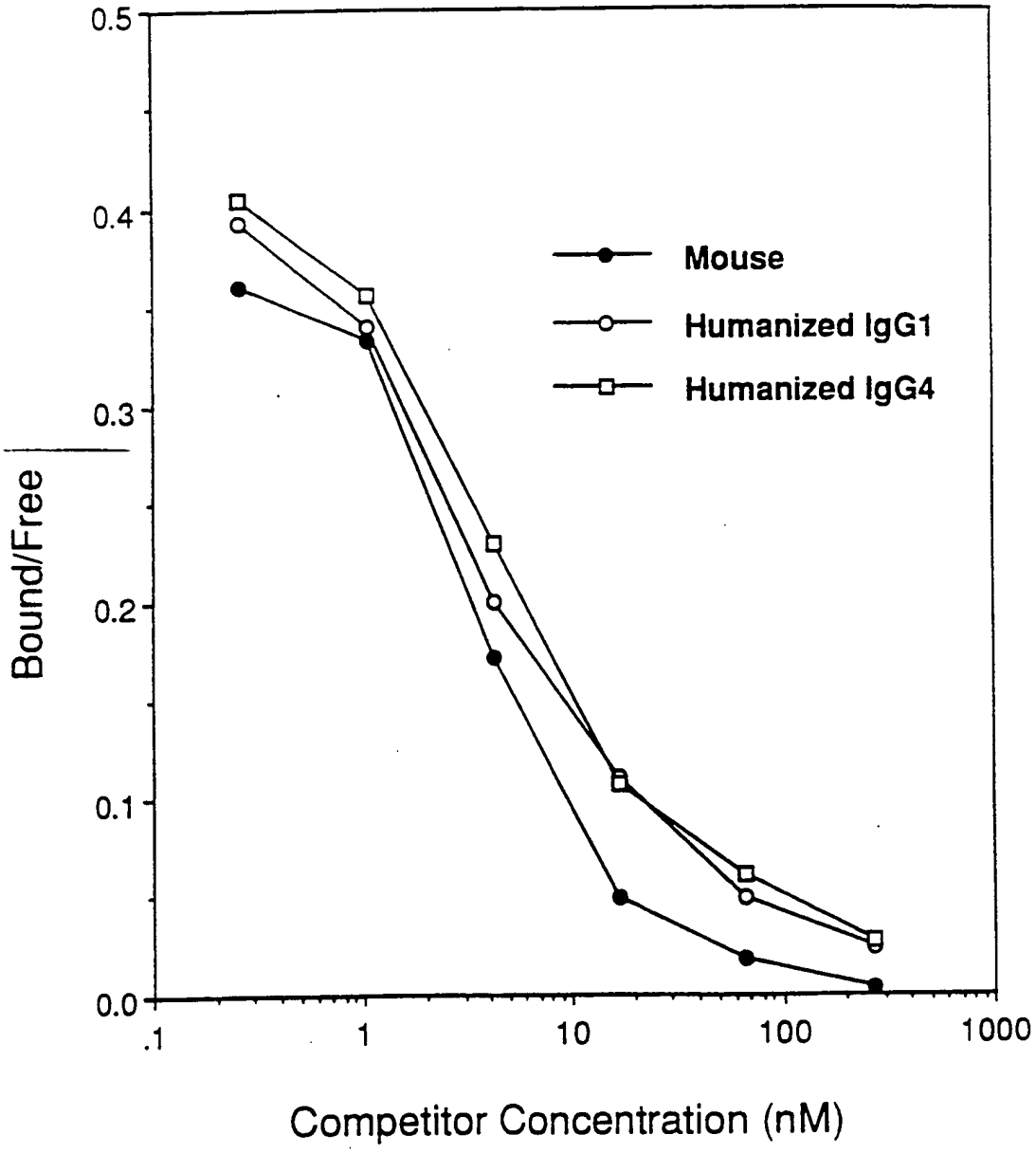
0.5
0.4
0.3
0.2
0.1
0.0
Bound/Free
.1
1
10
100
1000
Competitor Concentration (nM)
Mouse
Humanized IgG1
Humanized IgG4

Figure 4

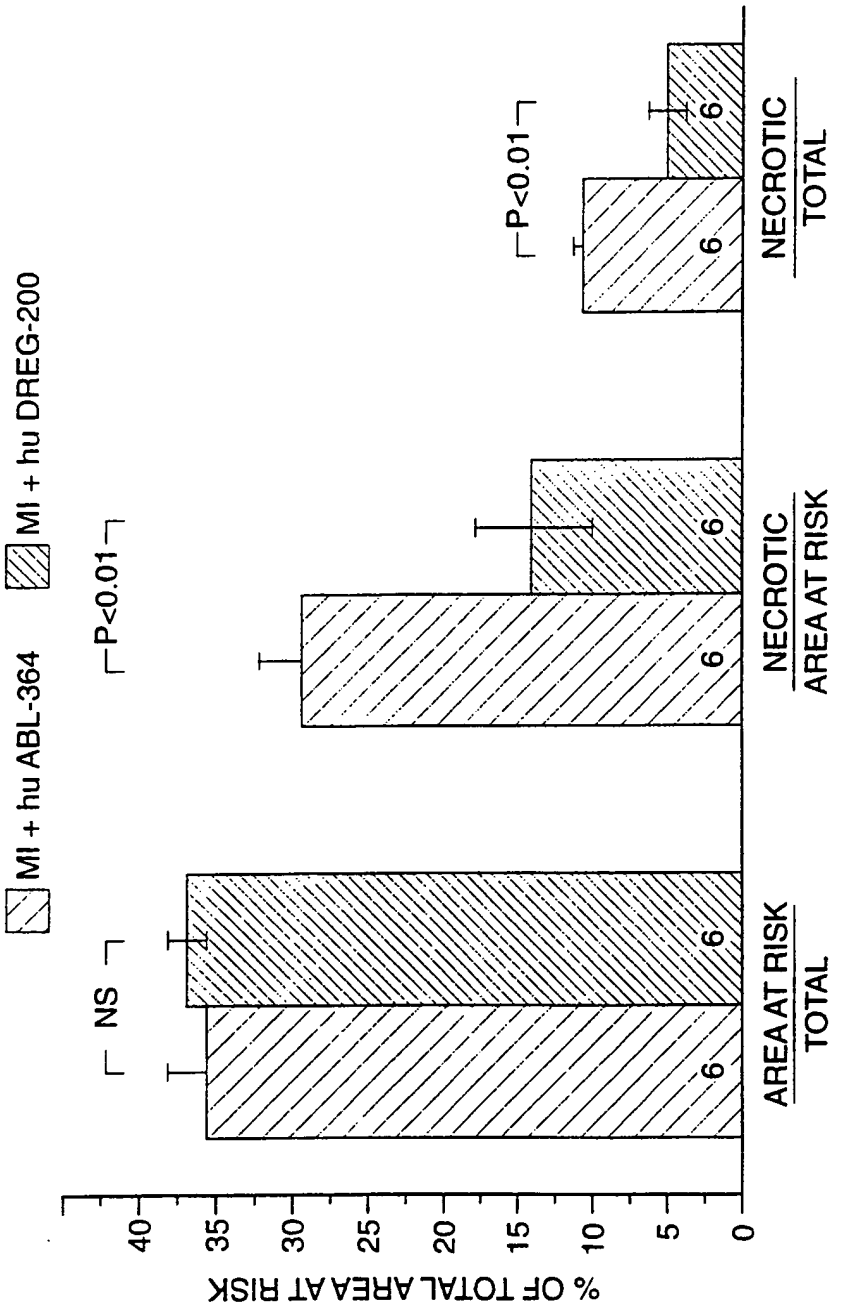
MI + hu ABL-364
MI + hu DREG-200
% OF TOTAL AREA AT RISK
0
5
10
15
20
25
30
35
40
NS
P<0.01
P<0.01
6
AREA AT RISK / TOTAL
NECROTIC / AREA AT RISK
NECROTIC / TOTAL

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11612

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Volume 78, No. 3 issued 01 August 1991, T.K. Kishimoto et al., "Antibodies Against Human Neutrophil LECAM-1 (LAM-1/Leu-8/DREG-56 antigen) And Endothelial Cell ELAM-1 Inhibit A Common CD18-Independent Adhesion Pathway In Vitro", pages 805-811, see entire document.	1-29
Y	Proc. Natl. Acad. Sci., Volume 86, issued December 1989, C. Queen et al., "A Humanized Antibody That Binds To The Interleukin 2 Receptor", pages 10029-10033, see entire document.	1-29
Y	EP, A, 0,440,351 (Law et al.) 07 August 1991, see entire document.	1-29